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Exposure of polystyrene microplastics induces oxidative stress and physiological defects in *Drosophila melanogaster*

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ABSTRACT

Background: Microplastics are emerging contaminants in aquatic and terrestrial environments. Here, we used the fruit fly (*Drosophila melanogaster*) as model organism to study the adverse effects of polystyrene microplastics (PS-MPs).

Methods: The computational study of styrene-protein interactome revealed enrichment of oxidative stress related pathways. Therefore, we tested the *in-silico* data by analysing the toxicity of PS-MPs via dietary exposures to 5g/l and 10 g/l doses on *Drosophila melanogaster*.

Results: At both of these doses there was significant increase in oxidative stress as revealed by lipid peroxidase assay. Furthermore, PS-MPs significantly reduced climbing ability of adult flies and distribution pattern of pupal positioning during development.

Conclusion: Overall, dietary exposures of PS-MPs caused toxicity in the fruit flies. This study establishes a baseline understanding of the impacts of PS-MPs to the fruit fly and motivates the need for further work focusing on naturally weathered plastic debris.

Keywords: *Drosophila melanogaster*; Polystyrene microplastics; Pupal position assay; Climbing assay; Oxidative stress; Lipid peroxidation assay; Protein-interactome; STITCH tool.

1. Introduction

Increasing plastic production and usage has magnified plastic pollution and has become one of the most serious environmental issues today (Rhodes, 2018) as it can take hundreds or thousands of years to decompose and in the meantime, wreak havoc on the environment. Plastics degrade and break into smaller pieces called microplastics (MPs) with a diameter of less than 5 mm (Law & Thompson, 2014).

MPs get transferred through the food chain from the environment into living organisms including humans (Ragusa et al., 2022). PS-MPs have been found to have toxic effects on human health (Hantoro et al., 2019) including toxicity at cellular level (Hwang et al., 2020). PS-MPs were found to accumulate in various organs and cause reproductive toxicity in male and female mice (Jin et al., 2021; Liu et al., 2022)**.** Since, the humans are also continuously exposed to different MPs, there is a pressing need to understand the effects of MPs on model organisms. *Drosophila* is an ideal terrestrial model for research due to its short lifespan, simple and inexpensive maintenance, and the fact that the *Drosophila* genome has been fully sequenced, more than 75% of human disease related genes have fly homologs. Therefore, we used *Drosophila melanogaster* as a model to study the effects of exposure to different concentrations of PS-MPs. The present study was undertaken to evaluate the effects of PS-MPs on *Drosophila melanogaster* development and physiology.

2. Materials and Methods

2.1. *Drosophila melanogaster* culture

Flies were trapped from the wild using plastic bottles with banana peel inside it. They were cultured on standard cornmeal medium. Single line culture of flies was maintained by transferring a gravid fly of *Drosophila*

melanogaster in separate cornmeal media containing bottles. The single gravid fly was allowed to lay eggs in the media. The hatched eggs produced larvae of same age group.

2.2. Preparation of polystyrene (PS) microplastic media

Polystyrene microplastic crystals were purchased from Hi-media (GRM8822-500G). Polystyrene crystals were kept in freezer in the refrigerator for 30-40 minutes. After this, polystyrene microplastic crystals were grinded using mixer grinder. To obtain fine particles of polystyrene microplastics this process was repeated multiple times and then it was sieved through the strainer (with 0.425 mm mesh size).

Powdered form of microplastic polystyrene crystals was later added in the media to prepare microplastic cornmeal media. Cornmeal media containing 5 g/l and 10 g/l of polystyrene microplastics was prepared for experiments.

2.3. Pupal position assay

Pupal position assay was performed following (Fauzi et al., 2020). First, several pairs of flies were transferred to culture bottles and left for ten days. On the tenth day, the position of each pupa was observed. Before observing the pupae, the distance between the surface of the medium and the bottle cap was divided into five zones of 2 cm each. Then, pupae in each zone were counted. If there was a pupa located in the line between the two zones, it was recorded to belong to the higher zone.

2.4. Climbing assay

Climbing assay was performed on the flies exposed to different media concentration following (Manjila & Hasan, 2018) in a glass cylinder of 50 ml. 20 flies from each experimental set were used to perform climbing assay. The total number of flies crossing 50 ml mark in 10 seconds were counted. Each assay was repeated thrice and average climbing ability of flies exposed to different media was calculated.

2.5. Lipid peroxidation assay

Lipid peroxidation assay was performed following (Ohkawa et al., 1979). 0.3 g of flies were weighed and homogenised by adding 2 ml of 0.5% Trichloroacetic acid (TCA) with the help of glass homogeniser. The homogenate was centrifuged at 5000 rpm for 15 minutes at room temperature. 1 ml of supernatant was transferred into clean and dry test tube. 2 ml of freshly prepared 0.5% Thiobarbituric acid in 20% TCA was added into test tube containing centrifuged sample.

Sample was incubated at 90 ^{o}C for 30 minutes in water bath. Solution was cooled at room temperature. Absorbance was taken at 532 and 600 nm. All readings were taken in triplicate.

MDA level was calculated by following formula:

MDA= (OD 532- OD 600X 100/1.56) X TV / [dw X 1000]

Where,

OD = optical density

TV = total volume of the sample dw = dry weight of sample

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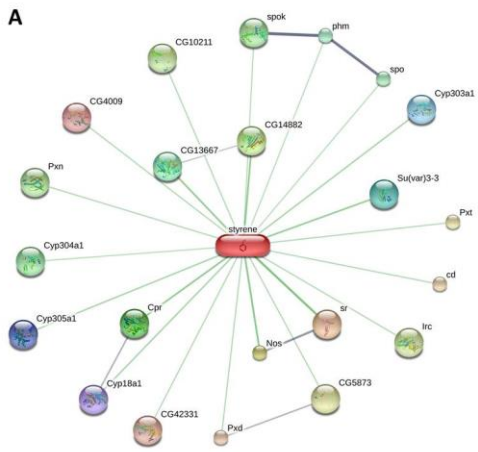
2.6. Polystyrene MPs interactome

To study the targets of PS, STITCH online tool (“search tool for interactions of chemicals”) (Kuhn et al., 2014) was used. This tool integrates information about interactions of a drug molecule with proteins and demonstrates the drug–target relationships. For creating the protein-protein network, the STITCH tool was adjusted for few parameters such as interaction score, which was set at medium confidence of 0.4 with no more than 50 interactors.

3. Results

3.1. PS-interactome revealed oxidative stress as the most enriched pathway in *Drosophila*

In order to understand the biological implications of the PS-interactome, we performed the GO annotation enrichment by STITCH tool (powered by PANTHER). The top 4 GO biological processes show oxidoreductase activity as shown in Figure 1.



B

Biological Process		
#pathway ID	pathway description	FDR
GO.0006979	response to oxidative stress	1.52E-10
GO.0042180	cellular ketone metabolic process	2.55E-05
GO.0006694	steroid biosynthetic process	8.59E-05
GO.0045455	ecdysteroid metabolic process	0.000239
GO.0055114	oxidation-reduction process	0.000461

C

Molecular Function		
#pathway ID	pathway description	FDR
GO.0016491	oxidoreductase activity	1.79E-20
GO.0020037	heme binding	5.89E-19
GO.0004601	peroxidase activity	1.23E-17
GO.0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	1.52E-15
GO.0005506	iron ion binding	4.39E-14

Figure 1. Styrene-protein interactome and GO enrichment analysis. (A) The drug-protein interactome was obtained by STITCH tool. Stronger associations are represented by thicker lines. The GO annotation showing the terms related to biological processes and molecular function. The false recovery rate (FDR) is also mentioned in the tables B and C.

3.2. PS induces oxidative stress in *Drosophila melanogaster*

Lipid peroxidation (LPO) assay revealed significant changes in Malonyl dialdehyde (MDA) production in the tissues of flies. Level of MDA was highest in 1g polystyrene microplastic treated flies (Figure 2).

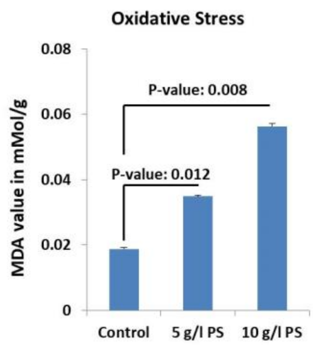


Figure 2. Analysis of oxidative stress in *D. melanogaster*. The flies were allowed to feed the normal, PS-MP (5 g/l) and 10 g/l. The MDA assay was performed to measure the oxidative stress.

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3.3. PS induces defects in pupal position and climbing ability of *Drosophila melanogaster*

Pupal position assay showed that while the control group of third instar larvae of second-generation flies could travel and pupate in zone E and F, those treated with polystyrene could not reach zone F. Very few of polystyrene treated flies (Figures 3A and B) were able to cross D and E zone.

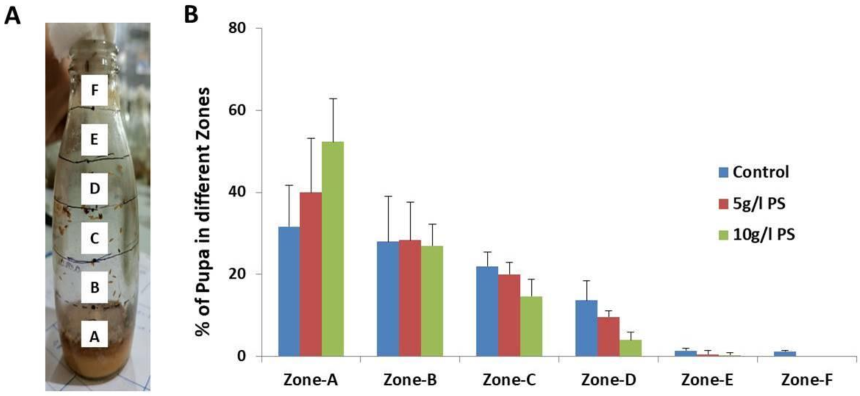

Figure 3. Analysis of pupal position of *D. melanogaster* in different conditions. The flies were allowed to feed the normal, PS-MP (5 g/l) and 10 g/l. (A) The distribution of zones has been diagrammatically represented. (B) The position of pupa in different zones were counted. Each assay was repeated at least three times and average pupal position in each zone was calculated.

Climbing assay showed that polystyrene interfered with the climbing ability of first- and second-generation flies (Figure 4). Climbing ability sharply declined in the flies cultured in media with 5 and 10 g/l PS-MPs.

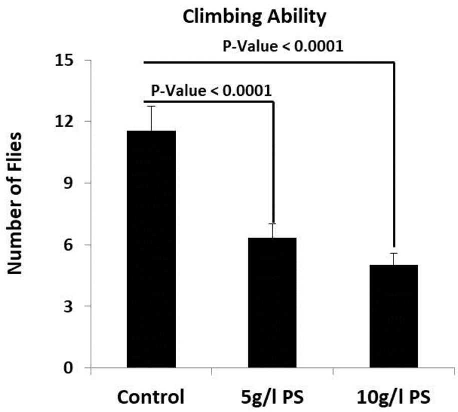

Figure 4. Analysis of climbing ability of *D. melanogaster* in different conditions. The flies were allowed to feed the normal, PS-MP (5 g/l) and 10 g/l. The 20 flies from each experimental set were allowed for climbing and their number was counted. Each assay was repeated three times and average climbing ability of flies of different media was calculated.

4. Discussion

In order to identify the mechanism of action of polystyrene on oxidative stress, we used *in silico* approach to study the molecular targets of this molecule. Here, we used STITCH webtool to analyse the drug-protein interactions. The PS-protein interactome data obtained from STITCH database shows interaction of styrene with *CYP303A1*, SR,

NOS, SU(VAR)3-3, CG4009, CG14882, CG13667, Cyp18a1 and Cyp305a1. *CYP303A1* belongs to the cytochrome P450 family, first found in *Drosophila melanogaster*, highly expressed in pupal stage. is indispensable for embryonic development and adult eclosion (Wu et al., 2019), SR proteins are master regulators of gene expression, if these functions are disrupted, developmental defects or disease may result (Long & Caceres, 2009). Nitric oxide synthases (NOS) catalyze the production of nitric oxide (NO) from L-arginine. Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis (Cinelli et al., 2020; Sharma et al., 2007). SU(VAR)3-3 is the *Drosophila* homolog of the human LSD1 amine oxidase (both are functionally conserved demethylases) and *Su(var)3-3* mutations suppress heterochromatic gene silencing (Holowatyj et al., 2015; Rudolph et al., 2007). CG4009 is a heme peroxidase, involved in exploiting the reduction of hydrogen peroxide to catalyze a number of oxidative reactions (Bailey et al., 2017). CG14882 is predicted to be involved in methionine biosynthetic process. CG13667 is predicted to enable FMN binding activity. Cyp18a1 encodes a cytochrome P450 enzyme (CYP) with 26-hydroxylase activity, a prominent step in ecdysteroid catabolism (Guittard et al., 2011). Cyp305a1 is predicted to enable heme binding and oxidoreductase activity (Li et al., 2019) .

We exposed *D. melanogaster* to different doses of PS-MPs to understand its toxicity. Here, the effect of toxicity of polystyrene was evident by the lipid peroxidation assay (LPO) where lipid peroxidation is a marker to measure oxidative stress which results from production of reactive oxygen species that damages the cell membranes, proteins and DNA (Ryter et al., 2007). Lipid peroxidation assay revealed the altered malonyl dialdehyde (MDA) production in the tissues of flies after the exposure of polystyrene. PS-MPs and micro-nanoplastics induced oxidative stress has been reported in different organisms by other workers also (Alaraby et al., 2022; An et al., 2021; Chen et al., 2022; El Kholy & Al Naggar, 2023; Feng et al., 2022; Khan & Jia, 2023; Kik et al., 2021; Meng et al., 2022; Mörbt et al., 2009; Ram et al., 2021; Schmidt et al., 2023; Turna Demir et al., 2022; Wang et al., 2019). Oxidative stress plays a major role in ageing and is associated with various neurodegenerative diseases (Luo et al., 2020; Maldonado et al., 2023; Warraich et al., 2020).

The pupal position assay was performed on the second-generation flies. The height of the pupal positioning indicated the climbing ability of the larvae before the fly entered the pupa phase (Wolfstetter et al., 2019). The height of the pupa is also a gravitational response and is included in the complex trait, which is determined by various other simple behaviours (Casares et al., 1997). In the present study it was found that very few of microplastic fed flies were able to pupate in the higher zones i.e., E and F zones of the bottle. The position of the pupa reflects the energy possessed by the larvae during the post feeding wandering phase**.**

Climbing ability data can be used as the parameter of motor function in *Drosophila* **(**Manjila and Hasan 2018**)**. Motor control involves the central nervous system to integrate various sensory inputs and translate them into specific movements that involve motor neuron and muscles (Maselli et al., 2014)**.** Furthermore, the presence of climbing defects in *Drosophila* also can be an indication of the existence of neurodegenerative disorders (Madabattula et al., 2015). In the present study it was found that there is notable suppression in the climbing ability of *Drosophila* in the second generation in the microplastic fed flies. The inhibited locomotion of adults could be attributed to the neurotoxic effects caused by Polystyrene. Locomotive defects resulting from toxic effects of

polystyrene can be a late onset symptom of neurodegenerative disease (Madabattula et al., 2015). Free radicals produced due to toxicity of polystyrene can be one of the reasons for the suppressed climbing ability.

5. Conclusion

This study provides a baseline understanding of the impacts of polystyrene microplastic exposure to the fruitfly, *Drosophila melanogaster*. Data suggests that polystyrene microplastic has significantly affected the pupal positioning and also caused oxidative stress. Polystyrene probably induced motor defects also, which was reflected in the reduced climbing ability of adult *Drosophila* also.

Microplastics are emerging contaminants, are passed through the food chain and accumulate in other living organisms including humans. They may cause harmful effects on human health. Further research focusing on naturally weathered plastic debris needs to be conducted. Moreover, microplastics may coexist with other contaminants in the environment and may behave as vectors that transport various toxic trace elements, including heavy metals and may cause greater harmful impact on human health.

Declarations

Source of Funding

This study did not receive any grant from funding agencies in the public or not-for-profit sectors.

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

The authors declare that they consented to the publication of this study.

Authors' Contributions

Himali Raj, Aditi Raj and Sumeet Ranjan did the experimental work and data analysis. Himali Raj prepared the original draft of the MS. Gajendra Kumar Azad did the *in-silico* analysis and edited the MS. Shahla Yasmin conceptualized, supervised, and edited the MS. All the authors read and approved the final manuscript.

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